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Wnt antagonist DKK-1 levels in systemic sclerosis are lower in skin but not in blood and are regulated by microRNA33a-3p

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Abstract

Background: Systemic sclerosis is an autoimmune skin disease which is associated with inflammation and resulting skin fibrosis. Myofibroblasts are the key cell type associated with the fibrosis but how they are differentiated is not clear. DKK-1 is a Wnt antagonist that blocks Wnt-mediated fibrosis and is reduced in fibrotic conditions. Thus, DKK-1 is a clear negative regulator of fibrosis in systemic sclerosis and its regulation is unknown. The aim of this work is to determine the levels of DKK-1 in serum and tissues of SSc and its regulation.

Methods: Skin biopsies were taken from early diffuse systemic sclerosis patients and healthy controls and DKK-1 measured by ELISA; serum was also isolated and DKK-1 quantified. DKK-1 was also measured by qRT-PCR. MicroRNA33a-3p was measured by TaqMan PCR. miR mimics and controls were transfected into dermal fibroblasts. Bleomycin mouse model was employed and compared to vehicle control treated mice, and gene expression was employed for DKK-1 and various extracellular matrix genes.

Results: DKK-1 is reduced in SSc skin and fibroblasts but is not reduced in the circulation in patients. MicroRNA33a-3p regulates DKK-1 levels epigenetically and is significantly reduced in SSc cells and whole tissue. DKK-1 is also reduced in the bleomycin mouse model and pro-fibrotic genes elevated.

Conclusion: DKK-1 is reduced in SSc cells and is regulated by miR33a-3p, and restoring DKK-1 levels through epigenetic means could be a therapeutic target in systemic sclerosis.

KEYWORDS

DDK-1, epigenetics, fibrosis, microRNA, systemic sclerosis

1 | INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune idiopathic connective tissue disease in which there are vascular pathology, inflammation, dysregulated cytokines and fibrosis.[1,2] SSc has the highest mortality rate of all the rheumatic diseases with a 10-year mortality rate of 23%-45%.[3] The disease predominantly affects women and depending on severity is classified as limited (confined to a single area) or diffuse, in which the fibrosis is widespread.[4,5] Although inflammation is key in the disease, the activation of the fibroblasts from a quiescent to an activated myofibroblast is considered a major pathogenic event in the pathogenesis of the disease. These myofibroblasts

secrete copious amounts of extracellular matrix primarily collagen and fibronectin and adapt a contractile phenotype that contracts the tissue, regardless of which organ is affected.[6,7] It is suggested that over half of all deaths in the developed world has a fibrotic component.

Although our understanding of the pathogenesis of the disease has substantially increased in the past few years, what governs the activation of the fibroblast remains unknown.[1] In recent times, the Wnt pathway has been identified as being a primary player in the disease.[8,9] The Wnt pathway is a development pathway that is critical in organ development and is highly expressed early in development then suppressed. Reactivation of development pathways such as the Wnt pathway in fibrotic diseases has recently been identified, and specific Wnt ligands are upregulated in SSc blood and skin tissue.[10] Beta-catenin which is considered a terminal part of the Wnt pathway is upregulated in SSc skin and lungs and localised primarily in the nucleus where it activates specific pro-fibrotic genes.[11,12] Indeed, overexpression of β-catenin in fibroblasts exacerbates bleomycin-induced fibrosis.[13] The Wnt pathway consists of the ligands that activate specific cell-surface receptors to initiate a response that recruits axin to the cell membrane and mediates the destabilisation of the β-catenin destruction complex, and β-catenin can then enter the nucleus and activate target genes. The pathway is also composed of a set of antagonists that can block specific Wnt signalling by trapping the ligand-receptor interaction or directly blocking the Wnt co-receptor.[14] It was found that one secreted Frizzled-related protein-1 (sFRP-1) is downregulated in SSc fibroblasts and is regulated by DNA methylation, and we also demonstrated this.[15] One other antagonist Dickkopf-1 (DKK-1) has also been found to be reduced in SSc cells.[15] The aim of this study was to ascertain the levels of DKK-1 in serum of diffuse SSc patients and the levels of DKK-1 in fibroblasts and its regulation.

2 | MATERIALS AND METHODS

Sixteen patients with early diffuse systemic sclerosis were recruited along with 16 gender-matched controls. All SSc patients fulfilled the new American College of Rheumatology/European League Against Rheumatism criteria for SSc;[16] 12 patients had anti-Scl-70 antibodies, and 4 had anti-RNA polymerase antibodies; and all had given informed consent, and this had been given institutional ethical approval. Female patients with early diffuse were 13 and 3 male early diffuse SSc, and mean age was 65. Serum was isolated after 20 mL of blood was removed by venepuncture. This was stored at −80°C until used for the ELISA.

DKK-1 was measured using a specific ELISA (R&D systems, UK) following the manufacturer's protocol and after development was read on a plate reader (Tecan) at 470 nm.

Skin biopsies were taken from lesions on the forearm using the punch biopsy 4 mm from 5 individual patients. The tissue was divided into two pieces; one was placed in TRIzol (Invitrogen), and RNA was isolated using manufacturer's instructions. The other piece was placed into tissue culture for isolated fibroblasts to grow out. These were all used at between passages 3 and 7 and cultured in Dulbecco's modified Eagle's media (DMEM) (Sigma, UK) + 10% (v/v) fetal calf serum supplemented with penicillin (100 U/mL) and streptomycin. Prior to any treatments, the cells were switched to serum-free DMEM the previous night and then cultured with or without Lipopolysaccharide (LPS) (1 ng/mL) (Invitrogen).

Healthy dermal fibroblasts were seeded into a twenty-four-well plate at 5×10^4 cells per well, and left to adhere overnight. Cells were then transfected with microRNA mimic miR33a-3p (Applied Biosystems) or scramble control at 75 nmol/L for 48 hours using HiPerFect transfection reagent (Qiagen), by mixing 75 nm with 5 μL of HiPerFect transfection reagent per well (of 24-well plate) and mixed and left at room temperature for ten minutes after which this was added to individual wells. After 48 hours post-transfection, media were removed and DKK-1 was measured by ELISA. Also, alpha-Smooth muscle was quantified by qPCR using specific primers: 5′-CAGGGAGTAATGGTTGGAAT-3′ (forward) and 5′-TCTCAAACATAATCTGGGTCA-3′ (reverse);18S 5′-CGAATGGCTCATTAAATCAGTTATGG-3′ (forward) and 5′-TATTAGCTCTAGAATTACCACAGTTATCC-3′ (reverse), using SYBR green (Sigma) and normalised to 18S. Data are shown as fold change compared to scramble controls. Soluble collagen was measured in the cell supernatant by Sircol assay (Bicolour,) as previously described.[17]

Isolated healthy control and SSc dermal fibroblasts were cultured, and then, RNA was harvested less than passage 3 using Qiagen miRneasy kits. qPCR was performed with TaqMan microRNA assay for miR33a-3p (id 4738831_miR) and normalised to housekeeping RNU44 (id 001094). Four individual donors were used, and all ran in triplicate.

qRT-PCR was performed on whole skin biopsies for DKK-1 using the primers human DKK-1:5′-GACTGTGCCTCAGGATTGTGT-3′ (forward), 5′-CAGATCTTGGACCAGAAGTGTCT-3′ (reverse). Axin-2:5′-CATGACGGACAGCAGTGTAGA-3′ (forward), 5′-TACTGCCCACACGATAAGGAG-3′ (reverse). Axin-2, forward 5′-CGG GAG CCA CAC CCT TC-3′ and reverse 5′-TGG ACA CCT GCC AGT TTC TTT-3′18S: forward 5′-GAA TGG CTC ATT AAA TCA GTT ATG G-3′, reverse 5′-TAT TAG CTC TAG AAT TAC CAC AGT TAT CC-3’ using SYBR green (Sigma, UK), from 1 μg of RNA. Data were normalised to the housekeeping gene 18S, and relative expression was quantified using the delta-delta CT method.[18]

SSc dermal fibroblasts from 4 donors were transfected with scramble or antagomiR to miR33a-3p (100 nmol/L) (Applied Biosystems) using HiPerFect transfection reagent as described above. After 46 hours, cells were lysed in RIPA buffer contain protease inhibitors and total protein assay was performed. 40 μg of total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V. This was subjected to transfer to nitrocellulose membrane and probed with collagen 1 (Abcam) at 1:1000 dilution overnight, and then, anti-mouse secondary antibody HRP linked was added at 1:10 000 dilution and then exposed for 1 minute. The same blot was stripped and reprobed

with β-actin (Cell Signaling) at 1:10 000 dilution for equal loading. Conditioned media from these scramble or antimiR33a-3p transfected cells were removed, and a TGF-β1 ELISA was used to quantify TGF-β1 (Quantikine, R&D systems) from four donors. All reactions were run in quadruplicate.

Bleomycin induced dermal fibrosis by subcutaneous injection for 4 weeks and was carried out as previously described.[2] Subcutaneous injections of 0.9% saline served as a vehicle control. All studies were performed with full ethical approval and under a UK home office licence. Four animals per group were used, and at the end of the experiment, skin biopsies were taken and placed in TRIzol. RNA was isolated using the manufacturer's instructions and cDNA synthesised. qRT-PCR was performed for with these primers: mouse collagen1A1 forward 5'-GAA GCA CGT CTG GTT TGG A-3', reverse 5'-ACT CGA ACG GGA ATC CAT C-3'; α-SMA: forward 5′-ATGCCTCTGGACGTACAACTG-3′, reverse 5′-CACACCATCTCCAGAGTCCA-3′; Axin-2 Forward: 5′-GAG TAG CGC CGT GTT AGT GAC T −3′; Reverse: 5′-CCA GGA AAG TCC GGA AGA GGT ATG −3′); TIMP-1 forward: 5'-GAC TAA GGC CTG TAG CTG TGC-3'; Reverse 5'-CTC GTT GAT TTC GGG GAA C-3'. qPCR was performed using SYBR green, and data were normalised to 18S housekeeping gene and relative expression quantified.

Mouse DKK-1 was measured by ELISA (R&D systems) following the manufacturer's instructions four mice per group and each mouse serum sample was run in quadruplicate.

Bleomycin treated mice and saline vehicle control treated mice were sacrificed at the end of 28 days skin biopsies were taken for haematoxylin and eosin (H&E) staining. Briefly, the tissue was fixed in formaldehyde. This was then placed in paraffin wax. Sections were cut at 5 μm thick, deparaffinised and stained with H&E and dehydrated and imaged using a light microscope at X20 magnification.

Statistical analysis was performed using Student's *t* test with *P* value equal or <.05 considered significant.

3 | RESULTS

The levels of serum DKK-1 in SSc diffuse patients were measured by ELISA and found to be not significantly different compared to healthy control patients (2.26 ng/mL SD = 0.08 vs 2.45 ng/mL SD = 0.096, *n* = 16 non-significant, *P* = .151 Student's *t* test; Figure 1).

We next examined the mRNA expression of DKK-1 in whole skin biopsies in SSc patients versus healthy controls and found that significantly reduced levels of DKK-1 in SSc skin compared to controls normalised to 18S mRNA 1 vs 0.44 HC v SSc *P* = <.0001 (*n* = 5; Figure 2A). Furthermore, culturing SSc dermal fibroblasts from the same tissue revealed significantly reduced DKK-1 levels in conditioned medium compared to control 2.45 ng/mL (SD = 0.41) vs 1.51 ng/mL (SD = 0.32) (*P* = .011 Student's *t* test; *n* = 4, Figure 2B).

Within the same skin biopsies, the levels of the Wnt target gene Axin-2 were significantly elevated compared to healthy controls (Figure 3A, 6.1 fold change SD = 0.4 5 vs 1.1 fold change, SD = 0.002, *P* = .001; *n* = 5 Student's *t* test).

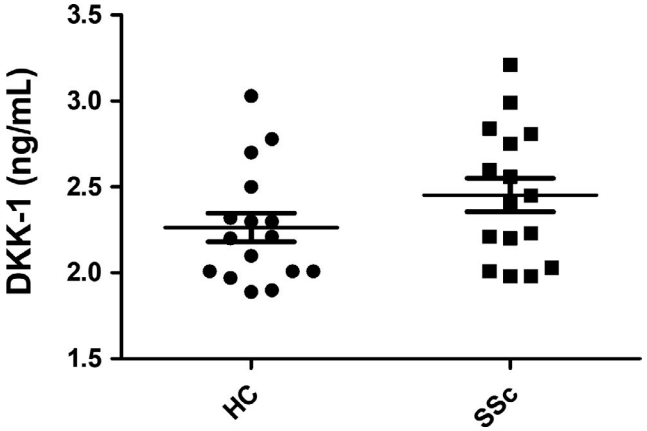


FIGURE 1 Serum DKK-1 is not significantly different in SSc patients. Serum DKK-1 was measured using ELISA in both healthy controls (HC) and SSc patients. No significant difference was observed, *P* = .151 Student's *t* test, *n* = 16. Main bar is the mean and SD

To examine what may be leading to the significant downregulation of DKK-1 in dermal fibroblasts, we incubated normal healthy dermal fibroblasts with the known fibrotic stimulator the TLR4 agonist LPS. However, incubation with LPS did not lead to a significant downregulation of DKK-1 expression (1.1 fold change treated vs 1 fold untreated; *n* = 4).

To examine if epigenetic regulation of DKK-1 is operative, we examined bioinformatically if DKK-1 can be targeted by microRNAs. Targetscan suggested that microRNA33a-3p can directly target DKK-1 leading to repression by binding to the 3'UTR, and Figure 3B demonstrates based on in silico analysis the binding site of miR33a-3p and positions 231-237 of DKK-1 3'UTR (bases in bold and underlined). Thus, transfection of miR33a-3p into normal dermal fibroblasts led to a significant reduction of DKK-1 expression (1.58 ng/mL SD = 0.03 vs 1 ng/mL SD = 0.04, *n* = 4, *P* = <.001 significant difference Student's *t* test; Figure 3C). Indeed, transfection of miR33a-3p into dermal fibroblasts leads to a significant increase in collagen release compared to concentration matched control mimics (*P* = .0002 significant difference Student's *t* test *n* = 4; Figure 3D). We also measured the expression of the myofibroblast marker alpha-smooth muscle actin and found this was not changed significantly (*P* = >.05 Student's *t* test; *n* = 4; Figure 3E). We next examined in isolated dermal fibroblasts from SSc patients and healthy controls the expression of miR33a-3p using TaqMan assays. We found that miR33a-3p is significantly elevated compared (3.3 fold) to healthy controls (*P* = .003; *n* = 4; Figure 3F).

Knowing that miR33a-3p is elevated in SSc patients' dermal fibroblasts and possibly targeting DKK-1 to mediate fibrosis, we used an anti-miR to miR33a-3p in SSc dermal fibroblasts to determine if this blockade would reduce collagen. Figure 4A indicates that anti-miR33a-3p reduced collagen 1 expression in these cells compared to scramble controls. However, no change in TGF-β1 levels was found after miR33a-3p inhibition (1.86 SD 0.11 vs 1.83 SD 0.17 ng/mL, *P* = .8 Student's *t* test, *n* = 4 Figure 4B).

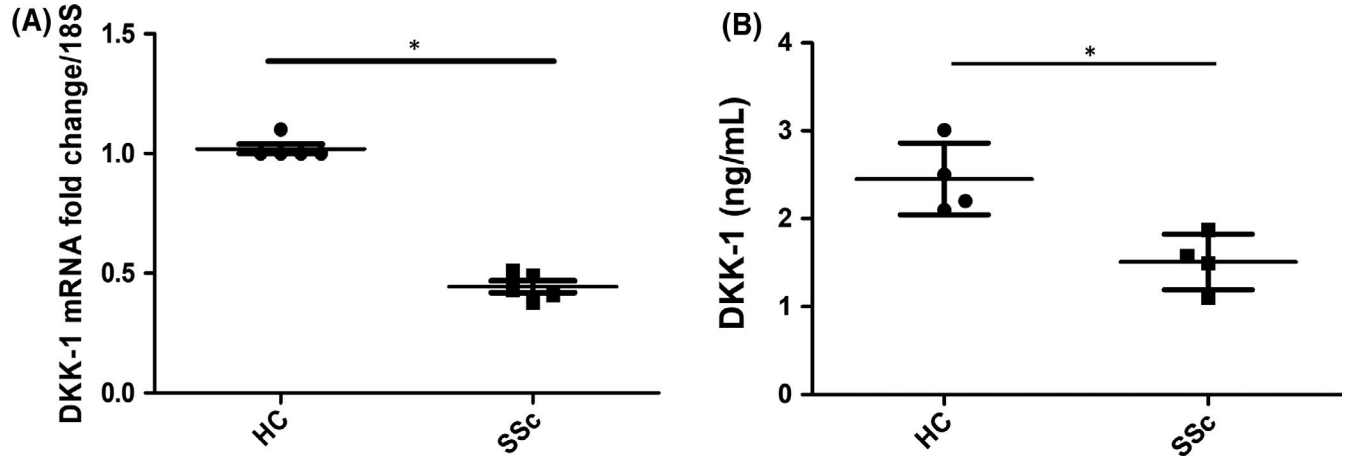


FIGURE 2 DKK-1 in skin tissue and fibroblasts is significantly reduced in SSc.(A) Whole skin biopsies were taken and analysed by qRT-PCR for DKK-1 expression and normalised to 18S. *Significantly reduced DKK-1 compared to HC, $P = <.001$ Student's t test, $n = 5$. (B) SSc dermal fibroblasts have significantly reduced DKK-1 levels. This was measured by ELISA and compared to HC, $P = .011$ Student's t test, $n = 4$

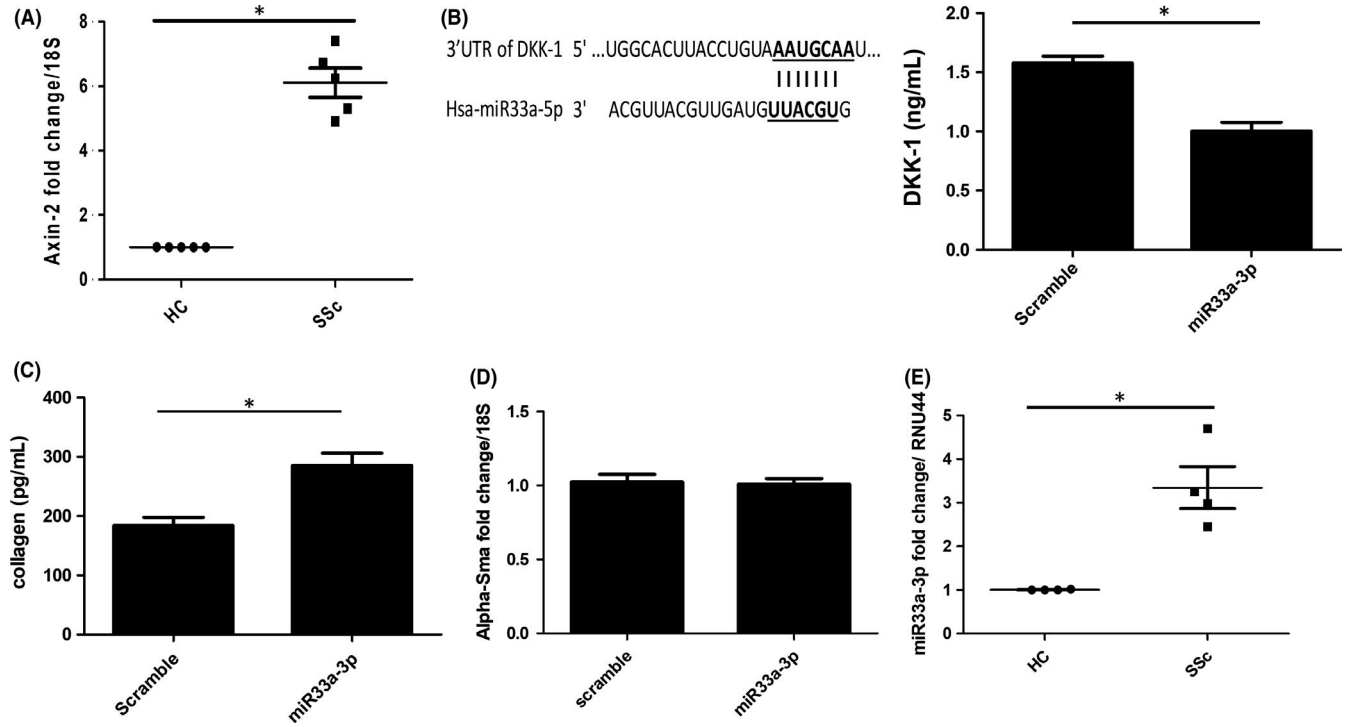


FIGURE 3 Wnt target gene Axin-2 is increased in SSc. (A) Whole skin biopsies were excised and measured for Axin-2 by qRT-PCR and normalised to 18S. *Significant difference compared to HC, $P = .001$ Student's t test, $n = 5$. Main bar is the mean and SD (B) Targetscan predicted binding sites of positions 231-237 of 3'UTR of DKK-1 and miR33a-3p. Bases are in boldface and underlined. (C) Transfection of miR33a-3p leads to reduced DKK-1 levels. Healthy dermal fibroblasts were transfected with miR33a-3p mimic (75 nmol/L) or matched scramble miR, and after 48 h, DKK-1 was quantified. * significant difference compared to controls, $P = <0.001$ Student's t test, $n = 4$. (D) Collagen was quantified by Sircol assay after transfection with miR33a-3p mimic or matched scramble control miR. * significant difference compared to scramble, $P = .0002$ Student's t test, $n = 4$ (E) Alpha-smooth muscle actin was quantified by qPCR after transfection with miR33a-3p mimic or scramble controls. Data are mean and SD and from 4 independent experiments. F, Elevated miR33a-3p in SSc fibroblasts. SSc isolated fibroblasts were quantified for miR33a-3p along with controls by qPCR. Data are normalised to RNU44 and shown as fold change compared to healthy controls. * significant difference compared to control, $P = .003$ Student's t test, $n = 4$

Finally, using the bleomycin model of skin fibrosis (Figure 5A), which is an inflammation-driven model, we could see that the whole skin gene expression levels of DKK-1 were reduced compared to vehicle treated mice 1.02 (SD = 0.02) vs 0.62 (SD = 0.05) fold change Vehicle v bleomycin treated ($P = .0003$; Student's t test $n = 4$; Figure 5B). Also, several pro-fibrotic genes were also significantly upregulated (Table 1). However, analysis of serum levels of DKK-1 demonstrated no significant difference (vehicle

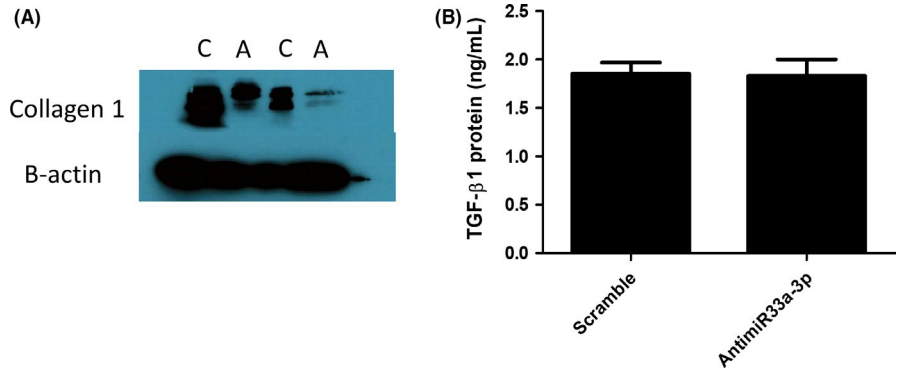
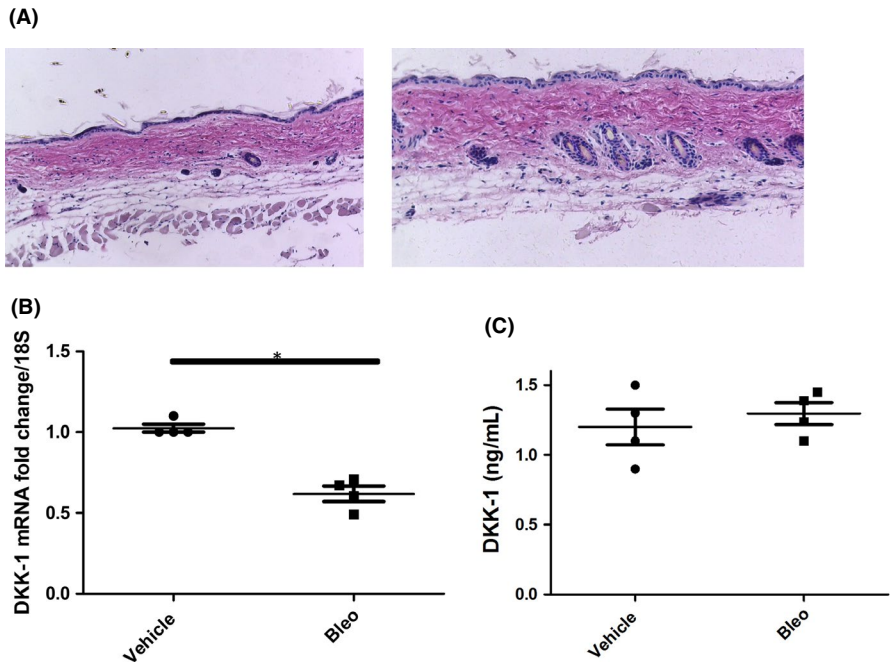


FIGURE 4 AntimiR reduced collagen in SSc fibroblasts. (A)Western blot from two independent donors early diffuse SSc after transfection with anti-miR33a or scramble controls. Top blot is collagen1 and β-actin is the loading control. C = scramble controls; A = antimiR-33a-3p. (B) TGF-β1 was quantified by ELISA in media of SSc cells after scramble or antimiR33a-3p transfection. No significant difference; Student's *t* test, $n = 4$

FIGURE 5 DKK-1 is reduced in the bleomycin model of fibrosis. (A) Representative H&E sections of whole skin left panel are vehicle control and right panel is bleomycin-treated skin. Magnification is X20. (B) Whole skin from mice exposed to bleomycin or vehicle control was analysed for DKK-1 by qRT-PCR. * significantly different compared to vehicle, $P = .0003$ ($n = 4$). (C) Serum DKK-1 levels are not significantly different in the bleomycin model of fibrosis. Serum DKK-1 was measured by specific ELISA after bleomycin or vehicle injection. No significant difference is evident across groups, $P = .771$; Mann-Whitney *U* test ($n = 4$)



1.2 ng/mL SD = 0.26 vs 1.29 ng/mL SD = 0.16, $P = .77$; Mann-Whitney *U* test n = 4 mice per group; Figure 5C).

4 | DISCUSSION

This study addressed whether the Wnt antagonist DKK-1 is reduced in SSc serum and skin. Surprisingly, we found that there was no reduction in the levels of DKK-1 in the sera of SSc patients. We had predicted that there would be a reduction in DKK-1 levels in the blood as this is an extracellular protein that mediates its effects via blocking Wnt signalling.

However, lower levels of DKK-1 were found in skin and isolated dermal fibroblasts from SSc patients. This is in keeping with the fact that the Wnt bona fide target gene Axin-2 is elevated in SSc cells also, indicating enhanced Wnt signalling in SSc.[13] It is known that Wnt signalling is important in tissue development but it is also been demonstrated to be elevated in SSc (7) and its inhibition reduces fibrosis in vivo animal models.[8,19] DKK-1 mediates its effects via binding to the Wnt accessory receptor LRP6,[14,20] and this leads eventually to β-catenin inhibition. We found reduced levels of DKK-1 in isolated dermal fibroblasts but not in the serum of SSc patients. To examine if Toll-like receptor signalling could reduce the levels of DKK-1, we incubated with LPS and could find no alteration in the levels of DKK-1. LPS is known to be pro-fibrotic in dermal fibroblasts.[1]

We sought to identify if DKK-1 can be regulated epigenetically as methylation of DKK-1 has previously been described,[15] and epigenetics is now thought to play a key role in SSc pathogenesis.[21,22] Using bioinformatics, we could find a putative binding site in DKK-1 for miR-33a-3p. MicroRNAs are small non-coding RNA that regulate gene expression at the post-transcriptional level by binding the 3'UTR of their target genes, and many microRNAs are dysregulated in SSc,[21,23,24] impacting on multiple processes. We could find that transfection of

TABLE 1 Gene expression changes in bleomycin-treated mice

Gene	Average fold change compared to vehicle control (SD)	*P* value (Student's *t* test) $n = 4$
Collagen1A1	3.6 (0.53)	<.001
Alpha-smooth muscle actin	5.9 (0.92)	<.0001
Axin-2	3.7 (0.98)	.00170
TIMP-1	2.9 (1.1)	.014

miR-33a-3p led to significant reductions in DKK-1 levels and also increased collagen release, suggesting that DKK-1 is a direct target of miR33a-3p. We, however, saw no change in alpha-smooth muscle actin expression. Transfection of anti-miR33a-3p into dermal fibroblasts to block miR-target interactions led to reduced collagen 1 expression in these cells. However, secreted TGF-β1 was not significantly different suggesting that this is independent of TGF-β pathways. We did not confirm that DKK1 is a direct target of miR33a-3p as we did not perform the 3'UTR luciferase assay; however, this is predicted to bind using bioinformatic analysis. miR33 is a critical microRNA that regulates cholesterol levels and is encoded within introns of sterol regulatory elements.[25-27] MicroRNA33-deficient mice are protected from experimental cardiac fibrosis,[28] and hepatic stellate cells, the myofibroblasts of the liver, have significantly elevated miR33.[29] Interestingly, microRNA-deficient mice have significantly reduced kidney fibrosis and application of a microRNA33 inhibitor reduced renal fibrosis in vivo,[30] suggesting this could be a therapeutic in SSc. We also demonstrated significantly elevated levels of miR33a-3p in SSc isolated fibroblasts compared to controls. What is regulating the increased miR33a-3p levels is not known; however in hepatic stellate cells, stimulation with TGF-β1 increased miR33a levels.[31] Elevated miR33a-3p levels may be used as a possible biomarker of disease given its direct association with DKK1 levels in dermal fibroblasts. Although caution must be used as the sample size here is small and conclusions regarding a possible biomarker need further validation in larger cohorts. Biomarkers in SSc are badly needed to help guide clinical decision-making.

We also confirmed that DKK-1 was reduced in an animal model of fibrosis, the bleomycin model along with pro-fibrotic genes including collagen and the Wnt target gene Axin-2. This is an inflammatory-mediated model of skin fibrosis and therefore suggests that inflammation, at least in the bleomycin model, could possibly be driving the reduction in DKK-1 expression. Analysis of sera from bleomycin mice revealed no significant differences between bleomycin treatment and vehicle control, which mirrors what we see in the SSc patients. Cell-intrinsic reductions in DKK-1 could be mediated epigenetically through a loop containing miR33a-3p.

4.1 | Conclusions

In conclusion, we demonstrate that the Wnt antagonist DKK-1 is reduced in skin and isolated cells but not serum of SSc patients and that in dermal fibroblasts this is regulated epigenetically by miR33a-3p. Strategies that reduce this microRNA could reduce Wnt signalling and fibrosis. Furthermore, miR33a-3p could be a possible biomarker in SSc skin.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JH, RS and SOR performed experiments. SOR designed the experiments and wrote the manuscript, and all authors have read and approved the final manuscript.

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